Deciphering Diversity at *er* Loci for Diversification of Powdery Mildew Resistance in Pea

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Abstract

Agricultural biotechnology aims to scrutinize the field crops which feed amongst half of the world's population by improving their agronomic traits using various biotechnological tools. Pea- an important cash crop, rich in nutrients, but frequently infected with powdery mildew (fungal disease) that destroy the whole crop which causes the economy loss for growers. We therefore, targeted this research to find the pathogen resistant pea lines and further decipher the diversity at *er* locus among resistant pea lines. Screening for resistant pea lines was done with *Erysiphe pisi* isolates (Genebank submission: KX45 5922.1) under net house and green house conditions. Molecular studies revealed that *Erysiphe* resistant (*er1*) gene was present in 40 lines out of selected 50 pea lines and the mutational character was conferred up to 36 genotypes with 11 haplotype groups. The haplotype (gene) diversity (Hd) was found to be 0.5571 ± 0.099 SD and the nucleotide diversity (Pi) was 0.0160 ± 0.0042 SD Majority of resistant lines (67%) occurred in Hap-1, other remaining haplotypes (Hap 2-10) having 33% resistant lines, each showing characteristic nucleotide substitutions with respect to reference PsMLO1 gene; genotypes from these divergent haplotypes can be used in pea resistance breeding to avoid genetic homogeneity and genetic vulnerability.

Introduction

During this global pandemic era, we are able to precise that the medical facilities become the priority for the life savior of the whole community. Although this is a universal talk, but to feed the whole community, agriculture plays an equal and important role in the well-being livelihood of the people across globally. Peeping back into history to till this pandemic situation, we can elaborate the role of various agricultural and horticultural crops to boost immunity against various diseases. E.g., Turmeric (Curcum), ajwain, ginger, garlic, among vegetables broccoli (anticancerous), lemon (vit c), all green leafy vegetables (rich in iron). The crops are not only known for their nutritive value; but also provide economy to farmers. These crops are grown in the whole world according to their geographical and climatic conditions. If we start our journey from North-west Himalayas, we pay our attention towards the Pea crop (*Pisum sativum*) which has been grown since many centuries for green pods and grains to meet the nutritional demands and economic upliftment of the growers. Nutritionally, pea crop comprises of protein (25%), slowly digestive starch (50%), sugars (12%), amino acids, carbohydrates, vitamins (A and C), calcium and phosphorus [1] along with lysine [2]. An interesting feature of this crop which increase its value as being a vegetable crop, it can be canned, frozen, dehydrated or dried and thus becomes a pulse crop. Being monumental one, several preventive measures has been taken for crop protection which occurred due to biotic and abiotic stresses. Powdery mildew of pea is one of the common biotic stresses, which is caused by the *Erysiphe pisi* which reduces the crop yield up to 50 per cent by affecting the quality and quantity of green pods and dry seeds of pea [3] [4]. Management of this drastic disease becomes compulsion because the pathogen not only affected the grain and pods, but also reduced pea foliage up to 33 - 69 per cent [5]. Banyal *et al.* 2005 [6] developed disease resistant cultivars by studying pathogenic variability of *E. pisi* among various pea varieties. These resistant lines having *er* (*Erysiphe* resistant) locus having MLO gene

(responsible for resistant mechanism in pea) which was detected using various molecular approaches. Present investigation therefore, carried out to find the presence of MLO gene among selected resistant cultivars of pea and deciphering diversity of *er* gene present among these resistant cultivars.

Results

Identification of test isolate

Morphological characteristics viz., hyphae, conidia, conidiophores, conidia size and conidiophore foot cells were studied on the detached leaves of host using stereo zoom microscope (Fig.1; Table 2). Attanayake *et al.* 2010 [7] described two groups of powdery mildew infected pea pathogen in combination of morphological and molecular characters. PCR amplification revealed an amplicon of approximately ~560bp, (Fig. 2) which was further gel purified and lyophilized before sequencing. BLAST analysis of the sequences of test isolates P-1 (from pea) and P-2 (from clover) indicated that both the strains were placed in the phylogenetic lineage occupied by the genus *Erysiphe* along with species, *pisi* and *trifolii*, respectively (Fig.2). The 18S rRNA sequence strain has been deposited in the NCBI GeneBank (Accession number KX455922 and KX455923, respectively).

Screening for resistant pea lines

Previously, screening was done and selected 3 resistant lines were crossed with JI-2302 (*er1*) and JI-2480 (*er2*) in 8 cross combinations viz., JI-2480 x Acacia, JI-2480 x PMR-10, JI-2480 x EC-381866-1, JI-2480 x Lincoln, JI-2302 x Acacia, JI-2302 x PMR-10, JI-2302 x EC-381866-1 and JI-2302 x Lincoln under net-house and green house and description of infection was observed [6]. Resistant was governed in maximum cultivars due to presence of *er1* gene (Table 3). We therefore, select *er1* gene for further studies.

Amplification studies

A total of 50 pea lines were used for RNA extraction (Fig.3). cDNA prepared from RNA was further amplified by specific primers mentioned in material and methods. To achieve this many times repeated PCRs were carried out in all the samples to standardize the protocol. Out of 50 lines, amplification was possible with primer 3F and 3R which produced 40 amplicons of variable size (250-300 bp) targeted that the *er1* gene was present only in these lines. (Table 4; Fig 3).

In silico analysis of gene sequences

BLAST N search for homology of all the sequences of *er1* gene corresponds to the gene present in *Pisum sativum* MLO1 (MLO1) mRNA, complete cds; homology queries values $\geq 90\%$ and E values near 0 for Nucleotide Blast analysis. Phylogenetic analyses separated the pea accessions into 3 groups. The major group A constituted by 32 accessions and the remaining 8 accessions were grouped as 6 and 2 in B and C group, respectively. Obtained tree was then saved to newick format and Fig Tree program was used for tree illustration (Fig.4) (<http://tree.bio.ed.ac.uk/software/figtree/>).

No. of haplotypes

A total of 11 haplotypes were obtained and the frequency of haplotypes ranged from 1 to 24. Hap-1 was the most abundant haplotype representing 24 genotypes including the reference genotype (FJ463618.1). The remaining haplotypes were represented by single genotype except Hap- 4 which is represented by 3 genotypes (Table 5). The Hap-1 having 23 genotypes showed 100 per cent similarity with reference genotype (FJ463618.1), hence does not have any base substitution w.r.t PsML01, whereas, Hap-2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 have 9, 5, 1, 6, 6, 16, 15, 14, 13 and 6 base substitutions, respectively.

Polymorphic sites

The analysis of polymorphic sites was carried out using DNAsp VI, and total of 36 sequences were used with a total of 198 variable sites. The 47 polymorphic sites included 18 Singleton variable sites, out of which 17 were with two variants and one was with three variants. There were 29 Parsimony informative sites out of which 26 were with two variants and three with three variants. The analysis of polymorphic sites was further studied in detail using multiple sequence alignment. Multiple sequence alignment was carried out in MEGA-(software) using the tool Clustal W [8]. Each haplotype of evaluated resistant pea genotypes were compared with the reference genotype for any site with replacement, deletion and addition (Fig. 5).

Haplotype diversity and Tajima's Test

Haplotype diversity was calculated using DNAsp VI where haplotype (gene) diversity was of 0.5571 and Nucleotide diversity (per site) of 0.01606 (Table 6). Tajima's test also found to be statistically significant with Tajima's D -2.09021 at $P < 0.05$. A median-joining network inferred from 40 set of sequences with 33 no. of active haplotypes was drawn. A value of zero was set for epsilon ($\epsilon=0$) to calculate sparse networks quickly, or incrementally. Maximum no. of mutations (29) was found at character 941 and least no. of mutations (1) were ranged at characters 992-1190 (Fig. 6).

Discussion

Till date, various research work has been carried out for the management of powdery mildew in pea plant [9] [10] [11]. Knowing the importance of this vegetable crop by exploring the literature we therefore, continued this research by deciphering the diversity at *er* loci in pathogen resistant pea lines [12] which was screened under invitro conditions [13]. Pathogen causing powdery mildew was collected from North-west Himalayas in which the maximum number of isolates were collected from Lahul area of trans-Himalayas [14] [15]. Molecular and morphological identification of test pathogen (from pea leaf and pea clover) corresponds to genus *Erysiphe* with species *pisi* and *trifoli*, respectively [16] [7] [17]. Screening was done under net-house and green house conditions where description of infection was recorded [6]. Selected resistant lines were crossed with the resistant carrier lines JI-2302 (*er1*) and JI-2480 (*er2*), respectively [11] [18] [19]. Maximum of resultant cultivars showed the character of JI-2302, hence governed by *er1* gene [20] [21]. Reviewing the available literature, it has been found that *er1* gene provides

a stable and resistance to *E. pisi* [22] [11] [23], thus can be used for breeding aspect. To reveal the presence of *er1* gene, RNA isolation was carried out the 50 lines of pea [21]. RNA was extracted manually and the cDNA sequences of their PsMLO genes at *er1* locus were targeted [24] [25] [21]. Out of four powdery mildew gene specific designed primers, primer PsMLO3F and PsMLO3R [26] successfully amplified the target *er-1* gene in 40 amplicons (250-300bp) which were further, purified and sequenced. Positive results indicated that PsMLO gene was expressed with primer 3F and 3R only after infection of *E. pisi* whether after 4 days / 8 days which can be related that this gene is inducible in nature [27]. BLAST N search for homology of all the sequences of *er1* gene corresponded to the reference gene present in *Pisum sativum* MLO1 (MLO1) mRNA, complete cds; homology queries values

$\geq 90\%$ and E values near 0 [28] [29]. Phylogenetic tree classified the pea accessions into 3 groups in which major group A constituted by 32 accessions and remaining 8 accessions were grouped as 6 and 2 in B and C group, respectively. In our analysis, largest clade comprises of major group A correspond $\geq 90\%$ of similarity with *Pisum sativum* MLO1 sequences. It can be supported that these sequences having MLO genes analogue of *er1* gene present in these accessions can be used for future breeding programs. A total of 11 haplotype groups were found where the frequency of haplotypes ranged from 1 to 24. Hap-1 was the most abundant haplotype representing 24 genotypes including the reference genotype (FJ463618.1) which revealed no base substitution w.r.t PsMLO1. The genotypes having *er1* gene grouped in Hap-1 represents the resistant alleles passed from resistant carriers linked together without any substitutions. In case of Hap-2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 have 9, 5, 1, 6, 6, 16, 15, 14, 13 and 6 base substitutions, respectively were found at MLO locus thus showing the diversity in the *er1* gene substitutions. Each haplotype of evaluated resistant pea genotypes were compared with the reference genotype for any site with replacement, deletion and addition. The genotypes from these divergent haplotypes can be used in pea resistance breeding to avoid genetic homogeneity and genetic vulnerability. Haplotype diversity was calculated using DNAsP VI A low value of *nd* (0.01606) revealed that these resistant lines can't be affected from environmental conditions. Tajima's test also found to be statistically significant with Tajima's D -2.09021 at $P < 0.05$. Network revealed the present of *er1* gene in great majority of lines shared identical haplotype with the reference PSML01 gene thereby suggesting that these lines has originated from a common ancestor.

Materials And Methods

Collection and identification of test pathogen

A total of 24 isolates of powdery mildew were collected from North-west Himalayas out of which maximum isolates were collected from 15 different locations of trans-Himalayan Lahul Spiti region. These were purified and maintained in greenhouse for further studies. The pathogen causing pea powdery mildew was identified on the basis of morphological characteristics viz., hyphae, conidia, conidiophores, conidia size and conidiophore foot cells. Further, polyphasic analysis of strain was done with 18S rRNA gene sequence. The sequence obtained was submitted to NCBI gene bank for accession number.

Screening for resistance

Screening for resistance against identified fungal pathogen was done from a panel of 310 pea lines comprising of exotic and indigenous germplasm collected from different sources (CSK HPKV Palampur, NBPGR New Delhi, PAU Ludhiana and IIPR Kanpur) were evaluated in net-house as well as on detached leaves under in vitro conditions [30]. The identified resistant lines along with susceptible ones were crossed with known recessive genes *er1* and *er2* present in JI-2302 (*er1*) and JI-2480 (*er2*) lines under greenhouse. Further, cultivars having resistance respective *er* gene were selected to determine the allelic diversity at *er* locus.

RNA extraction

A total of 50 pea lines were selected for RNA isolation using trizol method [31]. RNA was extracted from fresh leaves (without inoculation of *Erysiphe pisi*) and inoculated leaves (after 4 and 8 days of fungal inoculation), to check whether this gene is constitutive or inductive.

cDNA Synthesis and amplification studies

40 ug of RNA was used cDNA amplification using reverse transcriptase enhancer, 5x cDNA buffer, dNTP mix (5Mm each), verso enzyme as per the instructions recommended on Verso enzyme cDNA kit. PCR reaction mix was incubated at 42° C for 30 minutes. Further, reaction was terminated at 95° C for 2 minutes. For amplification of cDNA, PCR plates were filled with reaction mixture containing 5Xbuffer, 25mM MgCl₂, 10mM dNTPs, 0.5mM of each specific-designed PsMLO primer (Table 1), 5U Taq DNA polymerase with template cDNA. Amplification profile consisted 1 cycle at 95°C/5min; 37cycles at 95°C/30s, 50°C/ 30s and 72°C/1 min 20 sec; 1 cycle at 72°C/7 min; hold at 4°C/∞. The PCR products were separated on agarose gel (1.2%) and the targeted amplicons were purified and sequenced at the SciGenome Labs Private Ltd. Cochin, Kerala - INDIA.

In silico analysis of gene sequences

Homology of gene sequences were analyzed using online bioinformatic tools available in NCBI database in FASTA programme. BLASTN was used for sequence comparison on NCBI genomic database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) . Phylogenetic analysis was conducted in MEGA 5.0 [32] and genetic parameters such as haplotype diversity and total number of mutations, Indel polymorphism were calculated using DnaSP version 5.10 [33]. Network v 4.61 was used to construct Median joining (MJ) [34] network of the haplotypes

(<http://www.fluxus-engineering.com>).

Conclusion

For management of fungal diseases in crops, many strategies including conventional as well as non-conventional approaches are frequently used. From our research we identified the resistant cultivars in

pea crops which meet the demand of low and marginal farmers and reduces the use of chemicals in a controlled manner.

Declarations

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Statements

The authors declared that they have no conflict of interest in the publication

The datasets analysed during the current study are available in the NCBI Nucleotide repository,

<https://www.ncbi.nlm.nih.gov/nuccore/1131300078>,

<https://www.ncbi.nlm.nih.gov/nuccore/1131300079>

with accession numbers GenBank: KX455922.1 and GenBank: KX455923.1 respectively.

All ethical guidelines followed during collection of plant material from relevant mentioned institutes/Universities. Details of the plant material are already published (Rana *et al.*, 2013; Banyal *et al.*, 2015)

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Tables

Table 1 List of primers used for amplification

Primer Name	Sequence (5'-3')
PsMLO 1 FP	ATGGCTGAAGAGGGAGTTAAGGA
PsMLO1RP	CTAATTGCTCCCTAAGTGGCG CT
PsMLO2FP	CCTCGGAGAATTCTTGCTAC
PsMLO2RP	TCCACAAATCAAGCTGCTACC
PsMLO3FP	TCTGGCTCTTCACAGTGCTT
PsMLO3RP	TGTGGAAGCAAGAGGTTATGG
PsMLOEx5FP	ATGAGGAAGTGAAGACTTGGA
PsMLO15ExRP	GCTTTTGGCTGTGTGGTGCCAG

Table 2 Microscopic observations

Characters	Test Pathogen
Hyphae	Straight, branched, septate and hyaline
Conidia	Ellipsoid, cylindrical, ovoid shaped, hyaline and produced in chains
Conidiophores	Erect, straight, hyaline, arise vertically from the hyphae on the host surface
Foot cells	Cylindrical and decreased in width from base to the top
Cleistothecia	Spherical, gregarious, dark brown in colour and dispersed in mycelial web
Conidial (LXW)	25.5-52 x 11-18 µm
Conidiophores(LXW)	22-50 x 7-10.5 µm
Foot cells (LXW)	17.5-28 x 7-10µm
Cleistothecia diameter	87.5-133 µm

Table 3 Evaluation of F₁ population to study the allelic relationship with known *er* genes against pea powdery mildew caused by *Erysiphe pisi*

S.No.	Cross	Infection Type	Reaction Type
1.	JI-2480 (<i>er2</i>) x Acacia	2	Resistant (R)
2.	JI-2480 x PMR-10	3	Susceptible (S)
3.	JI-2480 x EC-381866-1	3	S
4.	JI-2480 x Lincoln	4	S
5.	JI-2302 (<i>er1</i>) x Acacia	3	S
6.	JI-2302 x PMR-10	2	R
7.	JI-2302 x EC-381866-1	2	R
8.	JI-2302 x Lincoln	4	S

Table. 4 No. of amplified pea lines

S.No.	Pea Genotypes
1.	1P-1287
2.	4P-995
3.	5P-1395-2
4.	7P-1301
5.	8P-1805
6.	9HFP-4
7.	10P-1806
8.	11P-1804
9.	12P-1820
10.	14P-144-10
11.	15P-1280-4
12.	16P-668-1
13.	17P-1707
14.	18P-48
15.	19P-1610-9
16.	20P-1436-9
17.	21P-1813
18.	22P-1377
19.	23P-1422-1
20.	24P1436-8
21.	25P1610-2
22.	26P-1506
23.	27P-1811
24.	28P-1440-20
25.	29P-1516
26.	30P-179
27.	31IPF-99-25
28.	32PKPMR-400

29.	39DPP-139-3
30.	40DPPMR-09-1
31.	42LFP-517
32.	43LFP-575
33.	45LFP-571
34.	48LFP-577
35.	50PB-29B
36.	51DPP-362
37.	52ACACIA
38.	55MR BIG
39.	56KMNR-894
40.	59DMR-11

Table 5. Frequency distribution of different PsMLO1 haplotypes detected amongst the resistant pea lines

Hap#	Frequency	Lines	No of base substitution(s)w.r.t. reference PsML01gene
Hap-1	24 (23+1)	FJ463618.1, P-1287, P-1820, P-1516, P-144-10, P-1707, P-1610-9, P-1377, P-1506, DPMR-09-01, P-179, P-KPMR-400, P-1440-20, LFP-575, LFP-571, ACACIA, MR_BIG, P-1280-4, P-1422-1, P-1610-2, P-1436-8, P-1806, P-1813, DMR-11	0
Hap-2	1	P-1804	9
Hap-3	1	P-995	5
Hap-4	3	HFP-4 P-48 KMNR-894	1
Hap-5	1	P-668-1	6
Hap-6	1	P-1811	6
Hap-7	1	IPF-99-25	16
Hap-8	1	DPP-139-3	15
Hap-9	1	LFP-517	14
Hap-10	1	LFP-577	13
Hap-11	1	PB-29-B	6

Table 6. Haplotype diversity and Tajma test

Haplotype (gene) diversity, Hd	0.5571
Standard Deviation of Haplotype diversity	0.099
Nucleotide diversity (per site), Pi	0.01606
Standard deviation of Pi	0.00462
Average number of nucleotide differences, k	5.20317
Number of polymorphic (segregating) sites	47
total number of mutations (eta)	51
tajima's d	-2.09021

Figures

Figure 1

Microscopic view of fungal cleistothecia

Figure 2

rDNA region amplified using *Erysiphe* specific primers (M: 1 Kb ladder; fungal isolate P-1 and P-2 along with tree phylogeny of P-1 :*Erysiphe pisi*, P-2: *Erysiphe trifolii*)

Figure 3

Figure 4

NJ tree based on MLO sequences. Posterior probabilities of the main clades above 0.93 (a) and bootstrap values in % (b) are indicated at the nodes (100). Designation includes pea lines number and name

Median joining network with median vector (red color dots), mutated characters (red color taxa) and sequence frequency (yellow color dots)

Supplementary Files

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